BACTERIOPHAGE PHENOMENA WITH STAPHYLOCOCCUS AUREUS

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A few months ago I began the study of staphylococcus "bacteriophage" processes, following at first the method by which Twort [1] observed these phenomena in 1914. After several weeks' work with unripened or "green" vaccinia virus from calves, a "lytic" strain was isolated, and most of the observations previously made by Twort and, in part, confirmed by Gratia,[2] were repeated. Meanwhile, the work of most of the observers, who have studied the d'Herelle phenomenon, and especially that carried on in this laboratory by Miss Kuttner [3] on lytic processes in gram-negative bacilli of the typhoid-colon group, seemed to indicate that the most important factor in the initiation of bacteriophage activity was contact with tissues in a state of disintegration, perhaps with tissue enzymes. For this reason, it seemed worth while to attempt the direct isolation of a lytic principle for staphylococcus from boils and other staphylococcus infections.

D'Herelle [4] himself recently reported a staphylococcus bacteriophage from a finger infection of 4 days' duration. Eight drops of the pus were put into 20 c c broth, incubated 24 hours, and filtered. The filtrate, after 5 generations, was active on albus, but not on aureus, both staphylococci having been obtained from the pus.

A total of 14 acute Staphylococcus aureus infections have been examined up to the present time. From 6 of these a bacteriophage specific for one or more strains of staphylococcus was isolated.

The following general technic was found most successful:

Sterile swabs containing pus from the boils were streaked directly on plain and blood-agar plates. Growths were examined on the next day and the individual colonies minutely searched for irregularities suggesting lytic changes.

In two cases to be noted in detail later, typical irregular colonies were found in the streaks made directly from the lesions in this way. Normal colonies were always fished to agar slants for the stock collection.

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1 Lancet, Dec. 4, 1915, pp. 1241-1243.
2 Proc. Soc. Exper. Biol. and Med., 1921, 18, p. 217.
3 Ibid., p. 222.
4 Le Bacteriophage, Monographies de L'Institut Pasteur, 1921.

Except in the two cases mentioned, direct streaks from the boils did not yield irregular colonies, and the lytic material was obtained only after the following manipulations. Swabs containing the pus were placed each in 10 c c of extract broth having a reaction of P_H 8, and then were incubated at 37 C. for about 4 hours. Previous experiments with vaccinia bacteriophage showed that longer incubations of the original material obscured the results. Recent observations render it questionable whether incubation is necessary; extraction by shaking and preservation in the icebox until use seems to be entirely satisfactory (see cases 13 and 15).

After incubation, the tubes were placed in the icebox over night or longer, as convenient, and before use were centrifuged and filtered through a Berkefeld candle. The filtrates so obtained were tested for "lytic" properties as described later.

Eighteen-hour broth cultures of 6 or more stock Staphylococcus aureus strains all recently isolated from boils were used. Each filtrate was tested against the autogenous strain, that is, against a strain derived from a normal colony of the same boil. In routine examination of the filtrates inhibition tests were found satisfactory; 0.1 c c of culture was added to each of 2 tubes containing 2 c c extract broth, P_H 8. Tube 1 was set aside as a control. To tube 2 was added 0.5 c c of the suspected lytic filtrate. With several strains of staphylococcus, a series of tubes with their controls were set up for the experiment. They were then incubated for two hours at 37 C.

During the short incubation period the tubes containing the filtrate were from time to time compared with the controls containing culture without filtrate for evidence of inhibition of growth. To prevent heavy growths, the tubes were removed from the incubator after 2 hours and left at room temperature over night and again observed at 18 hours.

Streaks of tubes and controls were made to plain agar plates at 2-hour and 18-hour intervals. Lytic action was recognized by characteristic "moth eaten" areas in the streaks, by irregular colonies with edges showing evidence of lytic changes, and by colonies with mottled surfaces. Whenever the action was marked, innumerable transparent or glassy colonies totally lacking in pigment were present, and there were few if any normal colonies. Occasional streaks showed no growth.

Two-hour streaks were generally more reliable than the 18-hour ones. This may be due to the fact that the normal resistant organisms predominate and overgrow the filtrates in cases in which small amounts

of the lytic principle are present. Complete inhibition of growth or subsequent clearing after growth does not mean sterility. These tubes will, on standing, again become clouded. This is due to the fact, now well known, that a culture is made up of 2 types of organisms, a sensitive and a resistant type. The sensitive ones are destroyed by the bacteriophage, the resistant survive and multiply slowly.

In the absence of any evidence of lytic action in the first generation, the experiment was carried through a second or a third generation, and so forth. Tubes containing the original filtrate and the control tubes after 18 hours were centrifuged one hour at high speed. Five tenths c c of each supernatant fluid was pipetted into tubes of broth containing 0.1 c c of corresponding 18-hour broth cultures of staphylococci, and the preceding routine examination was made. In one case marked "lytic" action did not take place until the fourth or fifth generation. The low concentration of the "bacteriophage" in the original filtrate was probably the reason for this.

The following cases of acute staphylococcus infections were examined, and a bacteriophage was obtained from each patient. The method of procedure in every instance followed exactly the routine technic which has been described in the foregoing. Successive steps in that procedure are indicated briefly with each experiment, and the results of each are summarized.

CASE 1. No. F 3104.—A man, aged 39, had a carbuncle on the back of his neck for about 5 days. This was incised and drained under local anesthesia. There was very little pus on the swab.

Direct streaks of the pus onto agar plates showed a pure growth of Staphylococcus aureus, but no colonies suggested lytic changes. The swab containing the pus was placed in 10 c c of broth P_H 8.0, and incubated for 3 days at 37 C. After preservation in the icebox over night the culture was filtered through a Berkefeld candle, and the filtrate so obtained was tested against 6 strains of Staphylococcus aureus for the presence of lytic material. The autogenous strain was lost.

The filtrate was active only against the laboratory stock strain of staphylococcus. A few colonies suggesting lytic action were obtained in the streaks made from the tube of the third generation. No inhibition of growth was noted. In the fifth generation a majority of the colonies showed irregularities. Partial but not complete inhibition of growth was evident. The experiment was continued in series through nine generations, but no further increase of lytic material was observed. The streaks still showed innumerable irregular colonies and many transparent ones, but complete inhibition of growth or subsequent clearing after growth did not take place.

CASE 2. No. F 2643.—A boy, aged 4, had a submaxillary abscess for 2 weeks with swelling and pain, and accompanied by a few eruptions on the face. It was incised and drained under ether anesthesia. There was about 0.5 c c of heavy pus.

The pus was streaked directly on to agar plates. Growths showed a pure culture of staphylococcus. The examination of individual colonies for evidence of lytic changes proved negative.

Ten c c of broth, P_H 8.0, containing the pus were incubated for 4 hours at 37 C. It was then preserved in the icebox for 3 days and filtered. The filtrate was tested against 6 strains of Staphylococcus aureus. The autogenous strain was lost.

The experiment demonstrated that the lytic material was active against only 2 of the 6 strains used, Nos. 1 and 4. A small amount of lytic action was apparent in the first generation. This was greatly increased in the second generation with both strains. However, total inhibition of growth or subsequent clearing after growth was never obtained even in the ninth generation.

CASE 3. No. F 4745.—A boy, aged 17, had a boil on his neck for 2 or 3 days. It was incised, and there was a moderate amount of pus.

A pure culture of Staphylococcus aureus was obtained from the pus. No colonies, however, suggested lytic changes. The swab containing the pus was placed in 10 c c of broth, P_H 8.0, and incubated for 4 hours at 37 C., preserved in the icebox for 3 days, and filtered.

The filtrate so obtained was tested against 7 strains of Staphylococcus aureus, including the autogenous strain from the same boil. Of these only 2 strains, strain 2 and the laboratory stock strain, were effected. Excellent lysis of both was obtained in the first generation.

As a further check on the autogenous strain, 4 other normal colonies from the direct streaks were fished to broth and the filtrate tested against each one of them. These experiments were carried on in series through 5 generations, but no lysis was observed in any instance.

CASE 4. No. 13.—A child, aged 8, had submaxillary abscesses, left and right, following an injury to the tongue; death in about 10 days; 3 c c of pus aspirated.

Direct streaks of the pus onto agar plates showed pure Staphylococcus aureus. There was a marked indication of lytic action throughout the streaks. The individual colonies were round but mottled in appearance. On restreaking these irregularities were duplicated. The heavy streaks contained a few clear areas and some colonies had uneven edges showing evidence of lytic changes.

Twenty-five hundredths c c pus in 10 c c of broth, P_H 8.0, was incubated 4 hours at 37 C., placed in the icebox for 4 days and filtered. Streaks made of the broth culture at the 2-hour and the 4-hour intervals showed typical irregular colonies and innumerable transparent ones. The filtrate was tested against 9 strains of Staphylococcus aureus, including the autogenous strain.

It was demonstrated that the filtrate was specific for the autogenous strain and 3 others, namely, 1, 4 and 11. Complete lysis, that is to say, a majority of irregular and transparent colonies in the streaks and a total clearing of the growths in the tubes after a few hours, was observed with each strain in the second generation. The autogenous strain seemed to be most susceptible.

In this experiment a comparison of the results obtained in 3 generations was made, as shown in table 1. It is interesting to note in the first generation experiments that there was no evidence of lytic action

in streaks or tubes. The second generation showed complete lysis of the 4 strains, as mentioned in the foregoing. This was considerably diminished in the third generation.

A parallel experiment was also carried out in which the 4-hour incubation period used in the preparation of the filtrate was eliminated. Instead, 0.25 c c of the pus was put into 10 c c of broth, P_H 8, and was shaken vigorously by hand for about 15 minutes, preserved in the icebox over night, and filtered. The filtrate so obtained was tested against autogenous strain 13. Extensive lytic action in the streaks and almost complete inhibition of growth was shown in the first generation.

TABLE 1

COMPARISON OF LYSIS IN THREE GENERATIONS

Strains of Staphylococcus aureus	1st Generation				2d Generation				3d Generation			
	Streaks		Tubes		Streaks		Tubes		Streaks		Tubes	
	2 Hrs.	18 Hrs.	2 Hrs.	18 Hrs.	2 Hrs.	18 Hrs.	2 Hrs.	18 Hrs.	2 Hrs.	18 Hrs.	2 Hrs.	18 Hrs.
Laboratory.....	—	..	H	H	—	—	H	H	—	—	H	H
Clinic 1.....	—	..	H	H	+	++++	H	Clear	+	—	H	Mod.
Clinic 2.....	—	..	H	H	—	—	H	H	—	—	H	H
Clinic 3.....	—	..	H	H	—	—	H	H	—	—	H	H
Clinic 4.....	—	..	H	H	+++	++++	V.F.	Clear	+	±	H	Mod.
Clinic 5.....	—	..	H	H	—	—	H	H	—	—	H	H
Clinic 10.....	—	..	H	H	—	—	H	H	—	—	H	H
Clinic 11.....	—?	..	H	H	++	++++	F.	Clear	+	—	H	Mod.
Autogenous 13.....	—?	..	H	H	++++	++++	Clear	Clear	++	±	H	Mod.

— = all normal colonies; + = an indication of lytic action; ++++ = many irregular colonies, very few normal ones; H = turbidity same as control; F. = faint turbidity as compared with control; V.F. = very faint turbidity; Mod. = moderate turbidity. The control protocols are not given. Streaks were always made at 2-hour and 18-hour intervals.

CASE 5. No. 14.—A woman, aged 26, had a carbuncle on the lower lip, accompanied by multiple abscesses on the face, staphylococcus septicemia. The lip infection had suffered a run down condition due to chronic general eczema. The patient had suffered with eczema since childhood with a series of boils each winter. She recovered.

Direct streaks of the swabs onto plain and blood agar plates gave a heavy hemolytic Staphylococcus aureus growth. The plates also showed several definite transparent areas in the streaks which suggested lytic action. These areas were fished from the center, and the material was streaked on agar plates. The growth showed innumerable irregular and transparent colonies. This proved for the second time that lytic material could be demonstrated in the streaks made directly from the lesion.

A filtrate was made in the usual way by incubating the swab containing the pus in 10 c c of broth, P_H 8.0, for 4 hours and filtering. Streaks from the broth culture again showed irregularities due to the action of a lytic principle.

The filtrate was tested against 11 strains of staphylococcus. Of these only 3 were susceptible to the action of the lytic material, namely, the autogenous

strain Nos. 14, 1 and 4. This action was very definite in the first generation and was only slightly increased in the second generation. The autogenous strain proved most susceptible.

A blood culture was taken on the same day. This showed on incubation a heavy growth of Staphylococcus aureus. A transplant was made from this to broth. After standing for two days at room temperature a partial clearing of the growth was noticed. Streaks were made immediately and showed colonies with typical irregularities. This proved that lytic material specific for that strain of staphylococcus was also present in the blood.

Fresh samples of blood and pus from the same patient were taken 5 days later. It was impossible to demonstrate again a lytic principle in either sample. Direct streaks showed regular normal colonies, and inhibition tests with filtrates carried through 2 generations were entirely negative.

A third blood culture taken 5 days later was sterile. The patient made a rapid recovery.

CASE 6. No. 15.—An infant, 10 days old, had cellulitis of the back of the neck, cerebral hemorrhage, cord infection and septicemia with secondary localization in the neck. Pus was taken 4 days after the swelling was first noticed.

Plate cultures made from the pus showed normal Staphylococcus aureus colonies, but no colonies suggested lytic action.

In this experiment 2 filtrates were made and compared. Into each of 2 tubes containing 10 c c of broth, P_H 8.0, 3 c c of pus were added. Tube A was shaken vigorously for 15 minutes, centrifuged and filtered on the same day. Tube B was incubated for 4 hours, preserved in the icebox over night, and filtered. A and B filtrates were then set up in parallel against 11 strains of staphylococcus, including the autogenous strain. Of these, only the laboratory stock strain was susceptible to the action of the lytic material in the 2 filtrates. Filtrate A was more active than B. Streaks from the tube containing filtrate A and culture showed a larger number of irregular and transparent colonies than streaks from the corresponding tube containing filtrate B. Also, filtrate A gave complete clearing after growth, filtrate B only partial clearing.

It is evident from the preceding experiments that a bacteriophage specific for one or more strains of staphylococcus can be isolated from acute staphylococcus infections. This again emphasizes the fact that when a pathogenic organism comes in direct contact with body cells, as is the case in an infection of any kind, a bacteriophage specific for that organism may be produced.

It is important to note that the filtrates which contained the lytic principle were not alike in their action on the several strains of staphylococcus used, that is, each one was active on a different strain or on a different group of strains and showed no action on the others. In other words, the different strains showed great variability to the action of a given lytic agent. Table 2 summarizes these observations.

In this table it is also shown that a staphylococcus bacteriophage isolated from vaccinia and carried through several generations was

specific for all the strains with which it came in contact. This proved that a strain which was resistant to the lytic material in the filtrates was not necessarily immune to all bacteriophage activity.

It was also difficult to increase the action of the bacteriophage isolated from the boils beyond the second or third generation. In successive generations it either remained constant or diminished in strength. Also, growth was seldom completely inhibited in the presence of the lytic agent nor were the cultures after growth completely cleared except in one or two instances. Experiments with the vaccinia bacteriophage, on the other hand, showed a marked increase through the fourth generation, at least, and did not deteriorate. A dilution

TABLE 2

REACTION OF VARIOUS STRAINS TO LYTIC AGENTS

Staphylococcus Strains	Filtrates						Vaccinia* Bacterio-phage	SPA Controls
	F 3104	F 2643	F 4745	No. 13	No. 14	No. 15		
Laboratory	+	..	+	..	..	+	+	—
Clinic 1	..	+	..	+	+	..	+	—
Clinic 2	..	..	+	..	..	..	+	—
Clinic 3	..	..	..	..	..	..	+	—
Clinic 4	..	+	..	+	+	..	+	—
Clinic 5	..	..	..	..	..	..	+	—
Clinic 10	..	..	—	..	..	..	+	—
(Autogenous of F 4745)								
Clinic 11	..	..	..	+	..	..	+	—
Clinic 12	..	..	..	..	..	..	+	—
Clinic 13	..	..	..	+	..	..	+	—
(Autogenous of 13)								
Clinic 14	..	..	..	..	+	..	+	—
(Autogenous of 14)								
Clinic 15	..	..	..	..	..	—	+	—
(Autogenous of 15)								

+ = typical lytic action present; — = no lytic action present.
* This lytic extract was originally derived from untreated calf vaccinia and used against the laboratory stock staphylococcus strain through several generations.

of one to one-million still caused inhibition of growth and complete lysis after growth.

Only two of the filtrates containing the lytic agent showed any action on the autogenous strain, that is, on the strain of staphylococcus derived from a normal colony of the same lesion. Material from these cases was probably taken before all the organisms susceptible to the bacteriophage had been destroyed. The other cases in which the action was negative probably represented a stage in which only the resistant organisms remained. Samples taken earlier in the course of the infection would no doubt have given positive results.

It has been impossible with so few experiments to judge during what period of an infection a bacteriophage appears, or how long it

persists. Case 14, in which lytic material was demonstrated in both blood and pus at one time and not at all a few days later, seems to indicate that the presence of a bacteriophage in an infected lesion may last for a limited period only, perhaps only until the susceptible organisms have all been destroyed.[4]

SUMMARY

The presence of a bacteriophage principle transmissible in series against Staphylococcus aureus has been demonstrated in the pus of a series of sixteen staphylococcus infections. In two of these the lytic principle was active against the autogenous strain as well as against other staphylococcus strains. In six others it proved lytic for one or more heterologous strains, but not for the homologous strain, in spite of repeated tests.

[4] Two cases which have been examined since this article was written showed the presence of a bacteriophage. The first was a case of empyema with a positive blood culture following an attack of influenza. A hemolytic Staphylococcus aureus was obtained, and a bacteriophage was isolated from both fluid and blood. It was not, however, specific for the autogenous strain. The patient did not recover.

The second case was a finger infection of 2 or 3 days' duration, from which a bacteriophage was obtained in the second generation. No lytic action on the autogenous strain was noted, even in the fourth generation.